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GENETIC VARIATION IN EASTERN WASHINGTON POPULATIONS OF *NAVARRETIA LEUCOCEPHALA* (POLEMONIACEAE) A VERNAL POOL ENDEMIC

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Abstract

We used Random Amplified Polymorphic DNA (RAPD) to investigate genetic variation in eight populations of the vernal pool endemic *Navarretia leucocephala* Benth. in eastern Washington. Five of the populations were subspecies *minima,* collected in Spokane County. The other three populations were subspecies *diffusa,* a recently described taxon known only from adjacent Lincoln County in eastern Washington. We also sampled a nearby vernal pool population of *N. intertexta* (Benth.) Hook., a closely related species whose range overlaps with *N. leucocephala.* Distances among the sampled pools ranged from a few tens of meters to 80 km. The populations differed in their levels of genetic variation, with proportions of polymorphic loci ranging from 10% to 55% and mean gene diversities from 0.05 to 0.30. Analysis of Molecular Variance (AMOVA) among the eight *N. leucocephala* populations showed significant spatial structuring, with 52% of the observed genetic variation due to differences between the Spokane County and Lincoln County populations. Within each county, 10% – 34% of the genetic variation was due to differences among populations. Estimates of Φ_{ST} indicated significant population differentiation among all populations of *N. leucocephala* (Φ_{ST} = 0.65), but the degree of differentiation varied with interpopulation distance. Populations 35–250 m apart were generally not differentiated. At a distance of 1100–1800 m, some populations showed significant differentiation and others did not. Populations 80 km apart were significantly differentiated. On a distance-based phenogram, *N. intertexta* separated clearly from the *N. leucocephala* populations, which also grouped by subspecies, suggesting that the morphological features used to differentiate the taxa are paralleled by genetic differences.

Key Words: Columbia Plateau, genetic differentiation, RAPD, *Navarretia,* Polemoniaceae, vernal pools.

Plants inhabiting ephemeral wetlands known as vernal pools are likely to show significant spatial structure in their genetic variation. Natural selection, restricted gene flow, and genetic drift can combine to create non-random distributions of genotypes at multiple spatial scales, with implications for the evolutionary trajectories of vernal pool species, and for efforts to preserve vernal pool biodiversity. Yet few experimental data exist on genetic variation within and among vernal pool populations (Elam 1998).

Vernal pools occur in regions with cool, wet winters and hot, dry summers. They form when winter rains fill depressions underlain by an impervious layer, then dry out during the spring and early summer, remaining dry for several months (Keeley and Zedler 1998). While vernal pools are most numerous in California, they also occur in the intermountain regions of several western states, including Oregon and Washington (Björk 1997), as well as Baja California, Mexico and areas of Argentina and Chile (Keeley and Zedler 1998). Pools generally show a highly clustered distribution, occurring in localized regions where the substrate and climate conditions allow their formation. Within these regions are "archipelagos" of pools a few meters to a few hundred meters apart, separated from other such archipelagos by tens to hundreds of kilometers (Holland and Jain 1981). Most vernal pool plant species do not occur in the intervening uplands, so this structured distribution of habitat imposes a strongly patchy distribution of populations, among which genetic variation may develop.

Because vernal pools are filled primarily through precipitation, local soil and hydrologic factors have a strong influence on the conditions experienced by vernal pool plants (Keeley and Zedler 1998). These different chemical and hydrologic environments are likely to select for different physiological and life-history characteristics, resulting in divergent selection pressures among pools. Many vernal pool plant species are endemic not only to vernal pools, but to specific subtypes of pools, differentiated by local climate factors, soil types, and topographic position (Stone 1990; Alexander and Schlising 1998; Bauder and McMillan 1998).

The potential for divergence among vernal pools is enhanced by the restricted nature of gene flow in many vernal pool plants. Leong et al. (1995) found that pollinators foraged occasionally between vernal pool patches 25 m apart, but rarely among patches 80–100 m apart. Using fluorescent dyes to track pollen movements in vernal pool *Limnanthes* species, Thorp (1990) found that 97% of the dye was distributed within 5 meters of the source plants,

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with occasional dispersal up to 13 meters. In *Limnanthes douglasii* R. Br. subsp. *rosea* (Benth.) C.T. Mason, a vernal pool endemic, 85% of plants received pollen from donors within 10 cm, and fewer than 5% received pollen from as far away as 80 meters (Kesseli and Jain 1982). Seed dispersal distances in vernal pool plants are also expected to be highly skewed, with most seeds falling close to the maternal plant (Zedler 1990). For example, vernal pool species in the genus *Navarretia* have indehiscent fruits, retaining their seeds within the capsule until the pools fill the following winter or spring. Swelling of the moistened seed coats bursts the membranous capsule, resulting in extremely localized dispersal (Spencer and Spencer 2003). Seeds of vernal pool plants may spread through wind-driven water movements, but transport among pools is likely to be limited (Jain 1978). On the other hand, some long-distance transport of seeds obviously occurs, given the broad ranges of many vernal pool taxa, and the discontinuous nature of their habitat (Schleidlinger 1981).

The genus *Navarretia* includes 12 species and subspecies that are more or less restricted to vernal pools and other ephemeral wetlands throughout the western United States, and a single species found in similar habitats in Chile and Argentina (Spencer and Porter 1997; Björk 2002; Spencer and Spencer 2003). Several taxa are widely distributed throughout the West. For example, *N. leucocephala* Benth. occurs from the San Joaquin Valley in California north through Oregon and eastern Washington, and east into Idaho and Utah (Hitchcock et al. 1964; Day 1993). A closely related species, *N. intertexta* (Benth.) Hook., also occurs throughout much of the range of *N. leucocephala.* In contrast to *N. leucocephala, N. intertexta* is more of a habitat generalist, occurring in vernal pools, but also inhabiting moist uplands and meadows (Day 1993a).

Day (1993b) identified five subspecies of *N. leucocephala* based on morphological characteristics; recently a sixth subspecies, *N. leucocephala* subsp. *diffusa* Björk, was identified from vernal pools in the Columbia Plateau of eastern Washington (Björk 2002). The most common subspecies of *N. leucocephala* in eastern Washington is *N. leucocephala* subsp. *minima* (Nutt.) Day, which is widely distributed in the region. Subspecies *diffusa* and *minima* both occur in similar habitats in Lincoln county, but their ranges in the county do not overlap. Subspecies *diffusa* is not found elsewhere in the state (Björk 2002). The degree to which the morphological differences between these subspecies reflect molecular genetic variation is unknown. In a recent phylogeny of the genus (Spencer 1997), an analysis of 22 morphological characters failed to distinguish among five subspecies of *N. leucocephala* (subsp. *diffusa* was not included). Adding sequence data from the ribosomal RNA internal transcribed spacer (ITS) region separated *N. l. minima* from the other four subspecies, but with little bootstrap support (Spencer 1997; Spencer and Porter 1997).

We used Random Amplified Polymorphic DNA (RAPD) markers to investigate genetic variation within and among populations of *N. leucocephala* from vernal pools in the Columbia Plateau of eastern Washington. RAPD markers (Williams et al. 1990) are a PCR-based indicator of genetic variation commonly used in studies of population genetics. RAPD markers are dominant, so heterozygotes cannot be directly distinguished from homozygotes at a particular RAPD locus. If populations are assumed to be in Hardy-Weinberg equilibrium, then the frequency of the "null" allele can be estimated as the square root of the frequency of the negative phenotype. If the assumption of Hardy-Weinberg equilibrium cannot be justified, then multilocus RAPD phenotypes can be treated as haplotypes, and genetic diversity and population differentiation can be estimated based on pairwise differences between individual haplotypes (Weising et al. 1995; Wolff and Morgan-Richards 1999).

Specifically, we sought to address the following two questions:

1) What is the current distribution of genetic variation among populations of *N. leucocephala* in eastern Washington?
2) Is the distribution of genetic variation concordant with the morphologically based subspecies designations of Björk (2002)?

Methods

In eastern Washington, vernal pools occur in three distinct "tracts", running roughly from northeast to southwest, following the paths of Pleistocene floods that scoured off surface soils and exposed basalt bedrock (Fig. 1). Outside of these tracts, soils are too deep or too well-drained to support vernal pool formation (Björk 2002). We sampled eight populations of *N. leucocephala* from vernal pools in Spokane and Lincoln Counties. The three Lone Pine Road populations (L1–L3) are subsp. *diffusa,* collected in the Swanson Lakes Wildlife Area in Lincoln County (47.6°N, 118.5°W), in the central, "Davenport" tract (*sensu* Björk 2002). The other five populations (SN1, SN2, SS1–SS3) are subsp. *minima,* collected in the Turnbull National Wildlife Refuge in Spokane County (47.4°N, 117.5°W), in the "Cheney-Palouse" tract (*sensu* Björk 2002). Pools within each group were 20–1600 m apart; the Lincoln County populations are approximately 80 km distant from the Spokane County populations. To compare intraspecific variation to interspecific variation, we also sampled a population of *N. intertexta* occupying a small vernal pool located between the two SN populations.

From six to ten entire plants were collected from each population, with no two collected individuals closer than two meters apart. DNA was extracted from approximately 50 mg of stem and leaf tissue

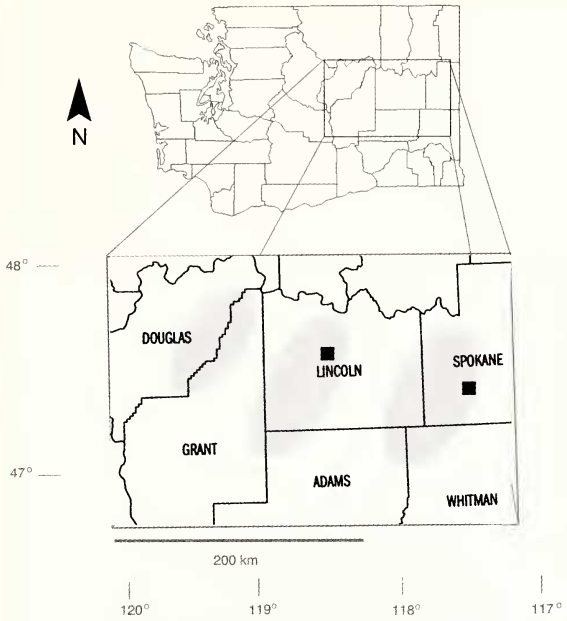

FIG. 1. Map showing locations of the *N. leucocephala* populations sampled for this study. Shaded areas indicate regions of vernal pool occurrence in Eastern Washington. Squares show locations of the sampled populations.

using the Wizard® DNA extraction kit manufactured by Promega Corporation (Madison, Wisconsin, USA), following the manufacturer's protocol. Genomic DNA was amplified with three 10-bp primers (Operon A1, A2, and A13), using RED-Taq® ReadyMix (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, 0.75 u Taq Polymerase; manufactured by Sigma-Aldrich, St. Louis, Missouri, USA), with 100 ng template DNA, and 25 pmol primer in a 25 uL reaction. Reaction conditions consisted of 3 min at 95°C, followed by 45 cycles of 1 min at 95°C, 1 min at 35°C, and 2 min at 72°C, ending with a three minute final extension at 72°C. Amplification products were separated in 1.8% agarose gels and visualized by staining with ethidium bromide. Gels were recorded using a digital camera with a Tiffen deep yellow filter (#15). Each individual was amplified at least three times with each primer, and only strong bands appearing in at least two runs were scored as present. The three primers produced 11 reliably scorable loci, all of which were polymorphic, and which produced 42 different multilocus haploypes in the 77 individuals sampled.

Statistical analyses were carried out using the software package Arlequin 2.0 (Schneider et al. 2000). For each population, we calculated the proportion of polymorphic loci, the mean number of pairwise differences among individuals across loci, and the average gene diversity (Nei 1987). Pairwise distances among all pairs of individuals (within and across populations) were computed as the number of different alleles between any two haplotypes. This matrix of individual distances was then used to estimate measures of genetic structure through an Analysis of Molecular Variance (AMOVA; Weir and Cockerham 1984). We partitioned total genetic variance into three covariance components: between counties (Lincoln vs. Spokane), among populations within a county, and within populations. The significance of these partitions was evaluated using permutation tests based on 1,000 randomizations of the data for each level of partitioning being tested (Schneider et al. 2000). We also analyzed each county's populations separately, partitioning variation into among- and within-population components.

The inter-individual genetic distances were also used to calculate pairwise estimates of genetic differentiation (Φ_{ST}) among all nine sampled populations (Excoffier et al. 1992; Wright 1965; Weir and Cockerham 1984). Significance of the Φ_{ST} values was evaluated using permutation tests based on 3000 randomizations, and using Markov-based exact tests analogous to Fisher's exact test for 2×2 tables (Raymond and Rousset 1995; Schneider et al. 2000). Estimates of gene flow between populations (Nm) were calculated using the formula:

$$\Phi_{ST} = \frac{1}{\frac{4Nmd}{(d-1)} + 1}$$

where N is the population size, m is the proportion of the population migrating, and d is the number of demes exchanging genes (Slatkin 1991). Because the actual number of populations exchanging genes is unknown, we calculated the estimates with $d = 2$, as if the two populations being compared only exchanged genes with one another. This is unlikely to be true, but the gene flow estimates were meant to be for comparative purposes only, and not as reliable measures of actual migration among populations.

Pairwise Φ_{ST} values were also converted to estimates of genetic distance using the formula $D = \Phi_{ST}/(1 - \Phi_{ST})$ (Slatkin 1995), which produces a measure proportional to divergence time between the populations. These estimates were used to create a distance-based tree using the method of Fitch and Margoliash (1967) in PHYLIP (Felsenstein 1993).

RESULTS

The RAPD markers showed significant genetic variation within and among populations of *N. leucocephala* in eastern Washington. The 77 individuals sampled possessed 42 different multilocus haplotypes, 32 of which were found in only a single population. Across populations, the proportion of polymorphic loci ranged from 10% to 55%, and the average number of pairwise differences between individuals within a population ranged from 0.47 to 3.33 (Table 1). Average gene diversity (the proba-

TABLE 1. SAMPLE SIZE (n), NUMBER OF MULTILOCUS RAPD HAPLOTYPES, PERCENTAGE OF POLYMORPHIC LOCI (P_p) MEAN PAIRWISE DIFFERENCES AMONG INDIVIDUALS, AND MEAN GENE DIVERSITY IN EIGHT POPULATIONS OF *NAVARRETIA LEUCOCEPHALA* FROM EASTERN WASHINGTON.

Population	n	Haplotypes (number unique)	P_p	Mean pairwise differences (±SD)	Mean gene diversity (±SD)
L1	6	5 (4)	36%	1.80 ± 1.20	0.16 ± 0.13
L2	6	6 (6)	55%	3.33 ± 1.99	0.30 ± 0.21
L3	8	7 (6)	60%	2.57 ± 1.54	0.25 ± 0.18
SN1	10	3 (0)	10%	0.47 ± 0.44	0.05 ± 0.05
SN3	9	7 (4)	45%	1.89 ± 1.19	0.17 ± 0.12
SS1	10	5 (2)	36%	1.47 ± 0.97	0.13 ± 0.1
SS2	10	5 (3)	36%	1.56 ± 1.01	0.14 ± 0.10
SS3	10	6 (4)	55%	2.38 ± 1.41	0.21 ± 0.14

bility that any two individuals drawn from the population will be different at a locus) ranged from 0.05 to 0.30. There was no geographic pattern to the distribution of within-population variation.

AMOVA results indicated significant genetic differentiation in *N. leucocephala* at all three levels analyzed (Table 2a). The majority of the variation (ca. 53%) was between the two counties, with most of the remainder (ca. 35%) found within populations. When the populations were analyzed separately by county, the majority of the variation was found to be within populations (Table 2b, c). In the Lincoln County populations, within-population variation accounted for 89.76% of the total, while in Spokane County, it accounted for 66.35%.

The overall Φ_{ST} value among all populations was 0.652 ($P < 0.001$), indicating a high level of differentiation. As in the AMOVA, much of this differentiation was due to differences between the two counties. When the two groups were analyzed separately, the Spokane County populations still showed significant differentiation ($\Phi_{ST} = 0.337$, $P < 0.001$), while differentiation among the Lincoln County populations was marginal ($\Phi_{ST} = 0.103$, $P = 0.077$).

On a pairwise basis, Φ_{ST} values indicated significant differentiation between most pairs of populations (Table 3). Permutation tests and exact tests gave slightly different results for the significance of these pairwise Φ_{ST} values. In particular, six Φ_{ST} values found to be significant under the permutation tests were not significant at the 0.05 level according to the exact test (Table 3). Of these, the three involving population SS3 had p-values between 0.055 and 0.066 according to the exact test; the three involving population SN3 had exact test p-values ranging from 0.11 to 0.26. When pairwise Φ_{ST} values were converted to estimates of migration rates, the vast majority were below 0.5 migrants per year. Among the most similar populations, estimates ranged from 1 to 4 migrants per year (Table 3).

In the distance-based phenogram, populations clustered together geographically, with the Lincoln County populations (L1–L3) grouped in a clade separate from the Spokane County populations

TABLE 2. RESULTS FROM ANALYSIS OF MOLECULAR VARIANCE (AMOVA) ON EIGHT POPULATIONS OF *NAVARRETIA LEUCOCEPHALA* FROM EASTERN WASHINGTON. Results are based on 11 RAPD loci treated as multilocus haplotypes. a) All populations combined. b) Five Spokane County populations only. c) Three Lincoln County populations only.

Source of variation	df	Sum of squares	% of variation	P
a) All populations				
Between counties	1	44.997	52.59	0.02
Among populations within counties	5	24.055	12.60	<0.01
Within populations	61	58.789	34.81	
Total	68	127.841	100.00	
b) Spokane County populations				
Among populations	4	19.24	33.65	<0.01
Within populations	44	35.46	66.35	
Total	48	54.69	100.00	
c) Lincoln County populations				
Among populations	2	4.82	10.26	0.08
Within populations	17	23.33	89.74	
Total	19	28.15	100.00	

TABLE 3. PAIRWISE Φ_{ST} VALUES (ABOVE DIAGONAL) AND CORRESPONDING ESTIMATED NUMBER OF MIGRANTS PER GENERATION, *NM* (BELOW DIAGONAL), AMONG EIGHT POPULATIONS OF *NAVARRETIA LEUCOCEPHALA* BASED ON 42 MULTILOCUS RAPD HAPLOTYPES. Bold type indicates Φ_{ST} values significantly different from zero at $P < 0.05$. Asterisks indicate Φ_{ST} values found to be significant under permutation tests, but not under exact tests.

	L1	L2	L3	SN1	SN3	SS1	SS2	SS3
L1		0.175	0.094	**0.841**	**0.735***	**0.718**	**0.715**	**0.625**
L2	1.18		0.058	**0.742**	**0.633***	**0.565**	**0.568**	**0.382***
L3	2.40	4.02		**0.708**	**0.585***	**0.542**	**0.552**	**0.423***
SN1	0.05	0.09	0.10		0.000	**0.485**	**0.444**	**0.519**
SN3	0.09	0.15	0.18	—		**0.393**	**0.361**	**0.428***
SS1	0.10	0.19	0.21	0.27	0.39		0.067	0.085
SS2	0.10	0.19	0.20	0.31	0.44	3.47		**0.220**
SS3	0.15	0.40	0.34	0.23	0.33	2.70	0.89	

(Fig. 2). Within the Spokane County populations, populations SN1 and SN3 are genetically indistinguishable, yet significantly differentiated from the three SS populations. As expected, the population of *N. intertexta* falls outside the clade that includes all eight *N. leucocephala* populations, despite the fact that it is less than 300 meters from population SN3, and less than 1700 meters from the SS populations.

DISCUSSION

Expectations for genetic variation in vernal pool plants will depend on the life-history of the species involved (Elam 1998). Among plants in general, annual species have been found to have lower within-population genetic diversity than perennials, based on allozyme (Hamrick and Godt 1989) or DNA markers (Nybom 2004). Summarizing 60 studies of wild plant populations using RAPD analysis, Nybom (2004) found an overall mean gene diversity of 0.22 ± 0.12. Thus the amount of genetic variation within populations of *N. leucocephala* (mean gene diversity: 0.18 ± 0.08) appears to be comparable to that for other annual plants in general.

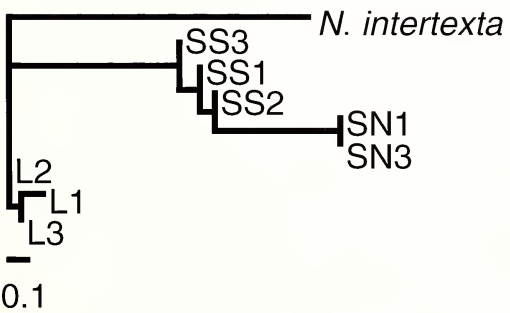

FIG. 2. Phenogram showing relationships among eight populations of *Navarretia leucocephala* and one population of *N. intertexta* in eastern Washington. Populations L1, L2, and L3 are subspecies *diffusa* from Lincoln County; populations SS1, SS2, SS3, SN1 and SN3 are subspecies *minima* from Spokane County, approximately 80 km distant. The population of *N. intertexta* was collected approximately 300 m from population SN3. Distances are Slatkin's linearized distances, calculated from 11 RAPD loci analyzed as multilocus haplotypes.

Within-population genetic diversity will also depend on the balance between opposing evolutionary and ecological processes. On one hand, isolation and genetic drift could lead to reduced within-population diversity. In contrast, self-incompatibility and long-lived seed banks could work to maintain significant diversity (Baker 1989; Hairston et al. 1996; Nunney 2002). All of the sampled *N. leucocephala* populations are quite large, with population sizes in the thousands of individuals. Thus, genetic drift is not expected to have a strong effect on within-population diversity, compared to other evolutionary forces. While the mating system of *N. leucocephala* is not known from experimental studies, pollen:ovule ratios suggest that it is primarily outcrossing (Cruden 1977; Plitmann and Levin 1990; Spencer 1997). Information on the seed bank is also unavailable, but the ephemeral, variable conditions in vernal pools favor the development of seed dormancy, and such dormancy is common among vernal pool plants (Elam 1998). These factors would tend to favor the maintenance of significant genetic diversity in populations of *N. leucocephala.*

The level of gene diversity we observed in *N. leucocephala* is comparable to that seen in RAPD studies of plants with predominantly selfing or mixed breeding systems. Nybom (2004) reported average gene diversities of 0.12 over ten predominantly selfing species, and 0.18 in eight species with mixed mating systems. Both of these values were significantly lower than the average of 0.27 for 38 predominantly outcrossing species (Nybom 2004). Thus, while pollen:ovule ratios may indicate a tendency for outcrossing in *N. leucocephala,* levels of genetic diversity suggest that selfing or mating among close relatives may be frequent in these populations. Because of the limited seed dispersal distances in *N. leucocephala,* plants often occur in clumps of many individuals in very close proximity. Members of these clumps are likely to be at least half-sibs, so pollinator movements among adjacent plants could result in significant inbreeding.

When all populations were considered together, *N. leucocephala* showed levels of population differentiation comparable to those found in RAPD studies of other annual plant species. Nybom (2004) reported a mean Φ_{ST} value of 0.62 across ten such studies, quite close to the value of 0.65 we calculated for *N. leucocephala.* But much of the differentiation we observed can be attributed to differences between the Spokane County and Lincoln County populations; separate analyses of these groups produce lower levels of differentiation. The Spokane County populations exhibit greater differentiation (Φ_{ST} = 0.337) than the Lincoln County populations (Φ_{ST} = 0.103), probably due to the smaller number and closer proximity of populations sampled in Lincoln County. In general, Φ_{ST} values based on RAPDs tend to increase with increasing interpopulation distances (Nybom 2004).

The Spokane County populations exhibit a level of differentiation similar to that seen in other RAPD studies of plants with mixed mating systems. Nybom (2004) reported an average Φ_{ST} of 0.40 for 18 such species, less than that seen for predominantly selfing species (mean Φ_{ST} = 0.65; N = 14), and greater than the average for outcrossing species (mean Φ_{ST} = 0.27; N = 73). Differentiation among the Spokane county populations is also higher than that found in most other studies of vernal pool plants, but this may be a result of the different markers used. Allozyme data from six vernal pool plant taxa, in two genera, showed F_{ST} values ranging from 0.083 to 0.176 (Elam 1998). A seventh taxon, *Limnanthes floccosa* Howell subsp. *californica* Arroyo had an F_{ST} value of 0.963, indicating extreme genetic differentiation among populations (Dole and Sun 1992; Elam 1998). The higher Φ_{ST} values we observed among *N. leucocephala* populations may result in part from greater variability of RAPD markers compared to allozymes. Hamrick and Godt (1989) reported an average F_{ST} value of 0.357 ± 0.024 for annual plant populations based on allozyme variation, compared to the average Φ_{ST} value of 0.62 reported by Nybom (2004) for annual plants using RAPD data.

The pairwise F_{ST} values observed in this study suggest that gene flow between pools a few hundred meters apart is generally sufficient to prevent differentiation. As a general rule, genetic drift can lead to differentiation between two populations if the number of migrants between them (Nm) is less than one per generation (Slatkin 1987). The three SS populations are all less than 100 m from one another, and show low to insignificant levels of differentiation and estimated Nm values between 0.89 and 3.5 (Table 3). Similarly, the three Lincoln County populations are separated by 35–250 m, and show no significant differentiation. The effects of intermediate distances on gene flow are equivocal. Populations SN1 and SN3 are 1300 m apart, yet they show no differentiation. In contrast, the SS populations lie 1000–1800 m distant from the SN populations, and pairwise comparisons among these pools show significant differentiation, with Nm values from 0.39 to 0.52 (Table 3). No obvious barriers to gene flow exist between the SN and SS populations, so the reason for this difference is unknown. The Lincoln County populations are separated from the Spokane County populations by a distance of 80 km; not surprisingly, gene flow estimates between these regions are quite low.

The pattern of genetic similarity depicted in Figure 2 is consistent with Björk's (2002) designation of the Lincoln County populations of *N. leucocephala* as a separate subspecies. Unfortunately, geographic distance and subspecies identity are confounded in our sampling, so firm conclusions about genetic support for the designation are not yet warranted. For example, we have no estimate of the amount of variation that might be observed between populations within a subspecies that are separated by 80–100 km. In addition, sampling of *diffusa* populations was quite localized, even within the restricted range of the subspecies. The samples used in this study were collected before the designation of *diffusa* as a separate subspecies, and were intended to sample *N. leucocephala minima* populations separated by a range of distances. Sampling of additional populations is hampered by the fact that most vernal pools in eastern Washington occur on private land, and landowners are generally reluctant to grant access.

The genetic differentiation observed among *N. leucocephala* populations separated by relatively short distances has implications for the conservation of vernal pool habitats and their associated species. Our SS and SN populations were separated by less than 2 km, yet we detected significant genetic differentiation among them. The large number of unique haplotypes found in each population also suggests that no single pool is likely to be representative of the genetic variation found across the larger landscape. Efforts to preserve vernal pool diversity should therefore focus on protecting populations throughout the larger regions in which they occur, rather than a few localized populations with significant numbers of individuals. This pattern in genetic variation is consistent with that seen at the community level in vernal pool floras. Within the California Floristic Province over 100 species of plants are known to be endemic to, or primarily associated with, vernal pools (Holland 1976; Keeley and Zedler 1998). Despite this diversity, individual pools generally contain only 15–20 plant species, indicating a high degree of variation in species composition from pool to pool (Holland 1976; Keeley and Zedler 1998). Consequently, vernal pool conservation efforts should be undertaken over large spatial scales, so as to capture the greatest amount of biological diversity, at the population as well as the community level.

Acknowledgments

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